PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Mehmet Toner et al.
Application No:	09/443,842 - 7776
Filing Date:	November 19, 1999
Entitled:	CONTROLLED REVERSIBLE PORATION FOR PRESERVATION OF BIOLOGICAL MATERIALS
Atty. Docket No:	22727-41

Group Art Unit: 1651
Examiner: V. Afremova

#13
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RULE 132 DECLARATION OF MEHMET TONER

I, Mehmet Toner, residing at 100 Pilgrim Road, Wellesly, Massachusetts, hereby declare as follows:

1. I received a Bachelor of Science degree in from Istanbul Technical University in Mechanical Engineering in 1982, a Master of Science degree in mechanical engineering from the Massachusetts Institute of Technology in 1985, and a Ph.D. in mechanical engineering from Harvard University-Massachusetts Institute of Technology Division of Health Sciences and Technology in 1989. After postdoctoral training in the same program from which I received my Ph.D., I was appointed an Assistant Professor of Surgery and Bioengineering at Harvard Medical School in 1990, and became an Associate Professor in 1995. In addition, I am Associate Director for the Center of Engineering in Medicine at the Massachusetts General Hospital. A copy of my Curriculum Vitae more fully explaining my qualifications, publications and appointments is attached as Exhibit A, however, it is further worth noting that I am President-

Elect of the Society for Cryobiology and will serve as President for the 2002-2004 term. I am also on the Editorial Board of the Journal of Cryobiology and Cryo-Letters, and I am an Associate Editor for the Journal of Biomechanical Engineering and Annual Reviews in Biomedical Engineering. I am an inventor on the above-referenced patent application.

2. I am familiar with the patent application at issue and, through this declaration, I hope to address the Examiner's comments made in the May 10, 2002 Office Action relating to the ability of a person of ordinary skill in the art to practice the claimed invention without undue experimentation.

3. The claimed invention relates to the preservation of nucleated cells having lipid membranes. The driving feature behind the claimed invention is that when cell membranes are reversibly porated and loaded with otherwise nonpermeable sugars for preservation, a number of unexpected and advantageous results can be achieved, such as the preservation of cells using only very low concentrations of sugar (resulting in simple loading procedures with no toxicity and no need to remove the cryoprotective agent), and the successful preservation of cells (namely nucleated cells) that otherwise have only been preserved using extremely complicated procedures, loading high levels of cyroprotective agents, and resulting in very little success.

4. There are four steps recited in the claims: 1) reversibly porating the target cells, 2) loading the porated cells with a bio-preservation agent to an intracellular concentration of less than or equal to about 1.0M, 3) preparing the loaded cells for storage (such as, for example, by cryopreserving), and 4) storing the cells so that they can be recovered in a viable state. In a preferred embodiment which I understand is also claimed, the bio-preservation agent includes a non-permeating sugar.

5. The purpose of the reversible poration step in the invention is to allow the cells to be loaded with the desired ***intracellular*** level. The application describes how reversible poration can be used to load the preferred non-permeable sugar (pages 4 and 5), as well as how reversible poration can be used to facilitate loading of conventional cryoprotective agents (page 8). The application then goes on to give guidance regarding preferred pore sizes (page 6), how to determine whether the poration scheme chosen works appropriately with the invention (pages 13 to 14), and the benefits of having the poration be reversible (page 26). The particular method of

reversible poration means nothing to the invention as long as it results in the desired results as expressed in the application, especially achieving the desired level of intracellular sugar.

6. While I believe that the use of membrane toxins to reliably form reversible pores of known size was the best mode of performing the method of my invention at the time this patent application was filed, it is by no means the only mode for performing the invention. In addition to the broad and general guidance provided in the application for the recited reversible poration step, prior art references cited in the application (and all cited references are incorporated by reference in the application) further describe poration and reversible poration methods that were then known to persons of ordinary skill in the art. These references include Russo M., "Reversible permeabilization of plasma membranes with an engineered switchable pore," Nature Biotech. 15, 278-282 (1997) provides a list of other available poration techniques, including reversible techniques such as electroporation and detergent treatment, with citations to references that describe the techniques; and Bayley, H., "Building doors into cells," Sci. Am. 277, 62-67 (1997) describes a variety of pore forming proteins having a variety of activation methods for switching the pores open and closed, including chemical, electrical and light based switches; both of which are cited on page 5 of the application.

7. In addition to the instructions provided within the four corners of the application, a person of ordinary skill in the art would have a number of other resources available to him or her to select and use a reversible poration technique for use with the claimed invention. For example, Professor James Weaver's chapter on "Electroporation of Cells and Tissues" in The Biomedical Engineering Handbook (CRC Press, Bronzino, J. (Ed.) (1995)) notes that "Electroporation is of growing interest because of its ability to rapidly and locally deliver molecules across bilayer membrane barriers" and that "Electroporation allows reversible or irreversible alteration of the cell membrane, as well as other lipid-based barriers in tissues, such that the barriers to ions and molecules are reduced within microseconds by several orders of magnitude." A person of ordinary skill reading my patent application would have, as of the filing date of the application, known that electroporation was one form of reversible poration that could be used with the claimed invention.

8. To show that electroporation could be used as the reversible poration technique within the claimed invention without undue experimentation, two post-doctoral fellows in my

laboratory carried out the electroporation and bio-preservation agent loading steps of the invention using electroporation techniques as described in papers published before the filing date of the application, and did so successfully. This work, described below, shows that reversible poration can readily be accomplished within the scope of the invention without undue experimentation.

9. The following data was generated to investigate various parameters of electroporation. In the first experiment, human foreskin fibroblast cells were exposed to different electrical pulses and the effect on cell poration was measured by examining the uptake of propidium iodide (PI), a fluorescent marker with a comparable molecular weight to trehalose (a non-permeable sugar). These cells were then exposed to electrical pulses. Fig. 1 shows a series of photographs in which the extent of PI uptake into human foreskin fibroblast cells was measured in relationship to the number of electrical pulses applied to the fibroblast cells. The data demonstrates that relative to the control sample (no electrical pulse), there was an increased number of cells that were fluorescent when the cell sample was exposed one electrical pulse. This shows that even with a single electrical pulse, the cellular membrane becomes porated such that the PI present in the culture medium is able to enter into the cells. Furthermore, the extent of PI uptake increases as the number of electrical pulses increases, with a maximum amount of PI uptake occurring with four electrical pulses.

10. To further characterize the effect of electroporation on the cell membrane, human foreskin fibroblast cells were exposed to different pulses, and different field strengths, and the uptake of PI by the cell was measured. Fig. 2 is a graph showing the change in fluorescence intensity of PI within a cell relative to an increase in the number of electrical pulses, and an increase in the field strength applied to the cells. The data shows that there was an increased uptake of PI by the cells as the number of pulses applied to the cells was increased. The data also demonstrates that there was an increased uptake of PI by the cells as the field strength was increased.

11. To asses the long term viability of cells after electroporation, 1×10^6 cells/ml human foreskin fibroblast cells were electroporated in electroporation buffer alone (control sample), or with electroporation buffer and 0.2M trehalose (test sample using the preferred sugar). The viability of the cells was measured by monitoring ethidium bromide/cyto uptake

after electroporation. Fig. 3 shows that 100% of the cells were viable before electroporation for both the control sample and the test sample containing trehalose. As the cells were exposed to an increasing number of electrical pulses, the viability of the cells in both the control and the test samples decreased, demonstrating that the electroporation was effecting the cell membrane. In the test sample, the trehalose entered into the cells upon electroporation, to a concentration needed to practice the invention. In fact, the overall viability of the cells containing trehalose was much higher than the control cells without trehalose. The data shows that trehalose enters into the cells upon reversible poration of the cell membrane by electroporation, and sustains cell viability thereby providing a protective effect on the cells.

12. In addition to Professor James Weaver's chapter in The Biomedical Engineering Handbook for electroporation (*Supra*), there were also a number of other articles that described this technique for reversible poration. A representative few of these articles are presented below. Thus, electroporation was a standard technique that was easily available to a person with ordinary skill in the art, and was routinely practiced at the time the invention was filed.

(i) Potter (1988) *Analytical Biochemistry* 174: 361-373. This review article provides examples of a number of different cell types that have successfully been reversibly porated using electroporation. These include mammalian cells, plant cells, unicellular organisms, as well as bacteria and fungi. The article also describes the introduction of proteins and small molecules by electroporation (*See* page 369, column 1).

(ii) Glogauer *et al.* (1992) *Exp Cell Res* 200:227-234. This article speaks to using electroporation as a method to gain access to the cell cytoplasm by transiently creating pores in cell membrane. Electroporation was used to introduce large-molecular-mass dextrans and proteins, as probes of the cytoplasmic compartment, into human fibroblast cells.

13. Also at the time the invention was filed, electroporation was by no means the only method available for reversible poration. Other reversible poration methods included using Glass Beads. For example, glass beads were used to reversibly porate the plasma membrane, as described in Fennell *et al.* (1991) *Arterioscler Thromb* 11:97-106.

This article describes how endothelial cells (ECs) were incubated with glass beads, to result in permeabilization of ECs. This poration of the plasma membrane allowed the introduction of macromolecules such as dextrans less than or equal to 152 kilodaltons, and immunoglobulins, as well as small, charged molecules (e.g., Lucifer Yellow) into the cell. The nonspecific permeabilization of the EC was transient and the integrity of the plasma membrane was reestablished.

14. Another method for reversible poration available at the time the invention was filed was by using pore-forming toxins. Examples of pore-forming proteins include various exotoxins produced by several strains of *Staphylococcus* and *Streptococcus*, some of which are described by Ahnert-Hilger *et al.* (1989) *Methods Cell Biol* 31:63-90. This article particularly focuses on pore-forming toxins such as α-toxin from *Staphylococcus aureus*, and streptolysin O (SLO) from α-hemolytic streptococci. The teachings of Ahnert-Hilger *et al.* show that α-toxins permeabilize cells for low molecular weigh substances, while SLO permeabilize cells for both high and low molecular weight substances.

15. Other examples of pore-forming proteins include the C5b-9 complex, as described in Bhakdi *et al.* (1978) *Proc Natl Acad Sci U S A* 75:5655-5659. This reference reports on the ability of purified C5b-9 complex, isolated from target membranes to become reincorporated into artificial lipid vesicles. A transmembrane pore appears to be created at the attachment site of the C5b-9 complex.

16. Yet another example of a pore-forming protein is perforin, a lytic protein isolated from cytolytic T-lymphocytes, as described in Masson *et al.* (1985) *J Biol Chem* 260:9069-9072. The isolated perforin polymerizes and inserts into lipid bilayers in the presence of Ca^{2+}, forming tubular structures with inner diameters varying from 6 to 16 nm.

17. One of ordinary skill in the art would be able to use the application's disclosure, in addition to the knowledge available in the art, to apply the invention to reversibly porating cells with any number of reversible porating techniques available at the time the invention was

filed.

18. In summary, the disclosure in the application, in combination with the knowledge available in the art for reversible poration, would enable one skilled in the art to perform the full scope of the claimed invention without undue experimentation.

19. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 10001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: June 10, 03

Mehmet Toner

Mehmet Toner, Ph.D.

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